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H740
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Y305 Y306 Y319 Y327 Y330 Y334 Y339 Y401 Y501
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Biochim.Biophys.Acta 1993,1156,313-320 Planta 1993,
191(1),137-142 Phytochemistry 1988,27, 3401-3403

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(54) alpha-1,4-Glucan lyase

(57) An enzyme, alpha-1,4-glucan lyase may be extracted from algae, especially Gracilariaopsis lemameiformis. The amino acid sequence, and DNA encoding said sequence, are also described. The enzyme may be used to prepare the sugar, 1,5-D-anhydrofructose (for use as a sweetener or anti-oxidant), by the treatment of an alpha-1,4-glucan, for example starch or dextrin, optionally in the presence of a hydrolase, preferably a glucohydrolase, in order to break bonds other than alpha-1,4-links. When isolated from algae, the enzyme may be purified by gel chromatography, especially a dextrin, preferably beta-cyclodextrin.

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Fig 1. a

A

1 2 3 4 5

kDa

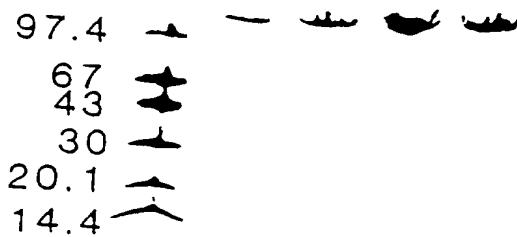
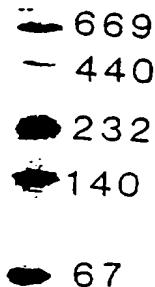


Fig 1. b

B

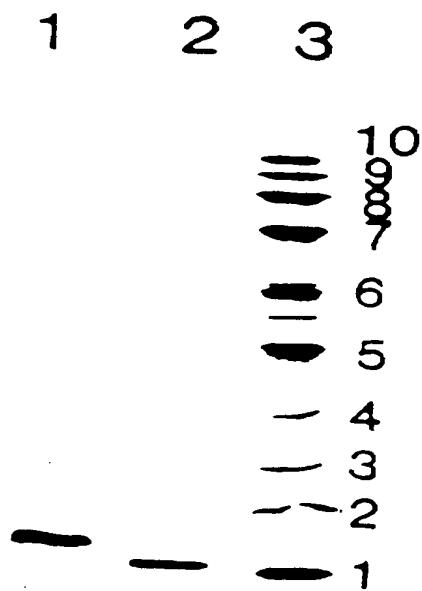
1 2 3 4 5

kDa



2/2

Fig 2



ENZYME

The present invention relates to an enzyme α -1,4-glucan lyase ("GL") and, in particular, its use to prepare 1,5-D-anhydrofructose ("AF") from substrates based on 5 α -1,4-glucan.

The present invention also relates to the use of a sugar, in particular 1,5-D-anhydrofructose ("AF") prepared by the method of the present invention, as an anti-oxidant, in particular as an anti-oxidant for food stuffs and beverages.

10

The present invention relates to the use of 1,5-D-anhydrofructose ("AF"), in particular AF prepared by the method of the present invention, as a sweetener, in particular as a sweetener for foodstuffs and beverages, preferably human foodstuffs and beverages.

15

FR-A-2617502 and Baute *et al* in Phytochemistry [1988] vol. 27 No.11 pp3401-3403 report on the production of AF in *Morchella vulgaris* by an apparent enzymatic reaction. The yield of production of AF is quite low. Despite a reference to a possible enzymatic reaction, neither of these two documents presents any amino acid 20 sequence data for any enzyme let alone any nucleotide sequence information. These documents say that AF can be a precursor for the preparation of the antibiotic pyrone microthecin.

Yu *et al* in Biochimica et Biophysica Acta [1993] vol 1156 pp313-320 report on the 25 preparation of GL from red seaweed and its use to degrade α -1,4-glucan to produce AF. The yield of production of AF is quite low. Despite a reference to the enzyme GL this document does not present any amino acid sequence data for that enzyme let alone any nucleotide sequence information coding for the same.

30 A typical α -1,4-glucan based substrate is starch. Today, starches have found wide uses in industry mainly because they are cheap raw materials.

Starch degrading enzymes can be grouped into various categories. The starch hydrolases produce glucose or glucose-oligomers. A second group of starch degrading enzymes are phosphorylases that produce glucose-1-phosphate from starch in the presence of inorganic phosphate.

5

AF has also been chemically synthesised - see the work of Lichtenhaler in Tetrahedron Letters Vol 21 pp 1429-1432. However, this chemical synthesis involves a large number of steps and does not yield large quantities of AF.

10 The chemical synthetic route for producing AF is therefore very expensive.

There is therefore a need for a process that can prepare AF in a cheap and easy manner and also in a way that enables large quantities of AF to be made.

15 Furthermore, anti-oxidants are typically used to prevent oxygen having any deleterious effect on a substance such as a foodstuff. Two commonly used anti-oxidants are GRINDOX 142 and GRINDOX 1029. These anti-oxidants contain many components and are quite expensive to make.

20 There is therefore a need to have a simpler and cheaper form of anti-oxidant.

Furthermore, sweeteners are often used in the preparation of foodstuffs and beverages. However, many sweeteners are expensive and complex to prepare.

25 There is therefore a need to have a simpler and cheaper form of sweetener.

According to a first aspect of the present invention there is provided an enzyme comprising at least any one of the amino acid sequences shown as SEQ. ID. No.s 3-4, or any variant thereof.

30

According to a second aspect of the present invention there is provided a nucleotide sequence coding for the enzyme of the first aspect of the present invention.

Preferably the nucleotide sequence is a DNA sequence.

According to a third aspect of the present invention there is provided a nucleotide sequence comprising a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of SEQ. ID. No. 1 or SEQ. ID. No. 2.

According to a fourth aspect of the present invention there is provided a method of preparing the sugar 1,5-D-anhydrofructose comprising treating an α -1,4-glucan with the enzyme α -1,4-glucan lyase, characterised in that enzyme is used in substantially pure form and wherein the enzyme is isolated from algae alone.

According to a fifth aspect of the present invention there is provided a method of preparing the sugar 1,5-D-anhydrofructose comprising treating an α -1,4-glucan with the enzyme α -1,4-glucan lyase characterised in that enzyme comprises at least any one of the amino acid sequences shown as SEQ. ID. No.s 3 - 4, or any variant thereof.

According to a sixth aspect of the present invention there is provided the sugar 1,5-D-anhydrofructose when prepared by the method of the present invention.

According to a seventh aspect of the present invention there is provided the use of a reagent that can increase the hydrophobicity of the reaction medium to increase the stability and activity of the GL enzyme.

25

According to an eighth aspect of the present invention there is provided the use of AF prepared by the method of the present invention as an anti-oxidant.

According to a ninth aspect of the present invention there is provided the use of AF prepared by the method of the present invention as a sweetener.

Preferably the enzyme is obtainable from algae, preferably it is obtainable from

Gracilariopsis lemameiformis.

Preferably the enzyme comprises at least any one of the amino acid sequences shown as SEQ. ID. No.s 3 - 4, or any variant thereof.

5

Preferably the enzyme is obtained from the expression of a nucleotide sequence coding for the enzyme.

Preferably the nucleotide sequence is a DNA sequence.

10

Preferably the DNA sequence comprises a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of SEQ. ID. No. 1 or SEQ. ID. No. 2.

15

Preferably if the glucan contains links other than and in addition to the α -1,4-links the α -1,4-glucan lyase is used in conjunction with a suitable reagent that can break the other links.

Preferably the glucan is starch.

20

Preferably a glucanohydrolase is used in conjunction with the α -1,4-glucan lyase.

Preferably the starch is used in high concentration - such as up to about 25% solution.

25

Preferably the hydrolase is at least one of pullanase or isoamylase.

Preferably the α -1,4-glucan lyase is bound to a support or, more preferably, is in a dissolved form.

30

Preferably the enzyme is isolated and/or further purified from algae alone using a gel that is not degraded by the enzyme.

Preferably the gel is based on dextrin or derivatives thereof, preferably the gel is a cyclodextrin - more preferably β -cyclodextrin.

Preferably the substrate is treated with the enzyme in the presence of a buffer.

5

Alternatively, preferably the substrate is treated with the enzyme in the presence of at least substantially pure water.

Preferably the substrate is treated with the enzyme in the absence of a co-factor.

10

Preferably the enzyme is used in combination with amylopectin or dextrin.

The terms "variant" or "homologue" include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid or amino acid from or to the respective sequence providing the resultant sequence has the respective ability to code for or act as an enzyme according to the present invention. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence has the ability to act as a promoter. With respect to sequence homology, preferably there is more than 80% homology, more preferably at least 85% homology, more preferably at least 90% homology, even more preferably at least 95% homology, more preferably at least 98% homology. Thus, the expression "substantial homology" covers homology with respect to structure and/or nucleotide components and/or biological activity.

25

The expression "contains any suitable codon substitutions" covers any codon replacement or substitution with another codon coding for the same amino acid or any addition or removal thereof providing the resultant enzyme has lyase activity.

30 In other words, the present invention also covers a modified DNA sequence in which at least one nucleotide has been deleted, substituted or modified or in which at least one additional nucleotide has been inserted so as to encode a polypeptide having the

activity of a glucan lyase, preferably having an increased lyase activity.

AF prepared by the present method was confirmed and characterised by ^{13}C NMR.

- 5 One of key advantages of the present method is that the sugar 1,5-D-anhydrofructose can be prepared in much larger quantities than before and by a method that is relatively easier and cheaper than the known processes. For example the sugar can now be prepared in amounts of for example greater than 100g - such as 500g - compared to the prior art methods when only much smaller amounts were and could
10 be produced - such as micro gram amounts.

Typical reactions that can be catalyzed by GL can be summarised as follows:

- 1). Amylopectin -----> AF + limit dextrin
15
2). Amylose -----> AF + limit dextrin

3). Dextrin -----> AF + glucose

20 In reaction 1), the ratio of the two products depend on the structure of amylopectin or the distribution of α -1,6-glucosidic linkages in the amylopectin molecules.

In reaction 2) and 3), the ratio of the products depends on the degree of polymerisation (DP) number of the substrate. In reaction 3 the ratio between AF and glucose depends upon the DP. For example if the dextrin contains 10 glucose units
25 the ratio AF:glucose would be 9:1.

- Another advantage of the present invention is that glucans that contain links other than α -1,4- links can be substantially degraded - whereas before only partial degradation was achieved. The substantial degradation of the 1,5-D-anhydrofructose precursor is one of the factors leading to the increased yields of 1,5-D-anhydrofructose.
30

Another advantage is that AF is a naturally occurring substance and therefore it has a potential for human purposes. For example, it can be converted to the antibiotic microthecin by AF dehydrase. Antibiotics are known for their uses in food bio-preservation, which is an important area in food technology. However, to date, the preparation of AF and also microthecin has had a number of disadvantages. For example, only small quantities could be produced. Also, the process was costly.

5 The present invention overcomes these problems by providing a larger production of and much cheaper production of AF and so also other products such as microthecin.
10 In this regard, it is possible to prepare gram to kilogram amounts of AF.

A further advantage is that the lyase is stable for at least one year at 4°C and can be lyophilized without loss of activity.

15 Another advantage is that the lyase produces AF directly from starches and does not need the presence of any co-factors.

Another advantage is that the enzyme can be used in pure water. This result is very surprising.

20 Based on the simple properties of the present lyase, one can expect that the production cost of AF will be comparable to that of glucose. This is especially advantageous that the present lyase does not necessarily require the presence of any co-factors which are generally very expensive.

25 In general α -1,4-glucans can be used as substrate for the enzyme.

As a preferred substrate, starch is used.

30 In a preferred process, soluble or gelatinized starch or starch hydrolysate are used. The starch hydrolysates can be prepared either chemically or enzymatically.

If an enzyme is used for the partial starch degradation the enzyme can either be added before the addition of the lyase or any other additional starch degrading reagent (such as the enzyme glucanohydrolase) which may be added simultaneously.

- 5 The lyase will convert the glucan to AF. The enzyme will attack the substrate from the non reducing end and leave only the reducing sugar unconverted. The residual glucose can be removed by known methods some of which have been described here.

10 Using the reaction described here pure AF can be produced and also in large amounts.

Thus, in one embodiment, the α -1,4-glucan lyase is purified from algae - such as *Gracilariaopsis lemaneiformis* - by affinity chromatography on β -cyclodextrin Sepharose, ion exchange chromatography on Mono Q HR 5/5 and gel filtration on 15 Superose 12 columns. The purified enzyme produces 1,5-anhydro-D-fructose from α -1,4-glucans.

The enzymes of the present invention convert amylose and amylopectin to 1,5-anhydrofructose.

20 Among the maltosaccharides tested, we found that the lyase showed low activity towards maltose, and lower activity to maltotriose and maltoheptaose with the highest activity to maltotetraose and maltopentaose. The enzyme showed no substrate inhibition up to a concentration 10 mg ml⁻¹ among these maltosaccharides.

25 The enzymes from the preferred source have been sequenced and the amino acid sequences are presented later.

Also presented later are the DNA sequences coding for the enzymes.

30 The present invention therefore describes a new starch degrading enzyme - namely a new α -1,4-glucan lyase. This is an enzyme that has been purified and characterized

for the first time.

As mentioned above, the present invention also relates to some specific uses of AF. In particular, the present invention relates to the use of 1,5-D-anhydrofructose ("AF"), as an anti-oxidant, in particular as an anti-oxidant for food stuffs and beverages.

Therefore according to the present invention there is provided the use of 1,5-D-anhydrofructose (AF) as an anti-oxidant.

10

Preferably AF is or is used in an edible substance.

Preferably AF is used in or as a foodstuff or beverage.

15 Preferably, AF is used in combination with another anti-oxidant.

Preferably the AF is prepared by the method according to the present invention.

The main advantages of using AF as an anti-oxidant are that it is a natural product, 20 it is likely that it is non-metabolisable at least for humans, it is easy to manufacture, it is water-soluble, and it is generally non-toxic.

A preferred embodiment the present invention therefore relates to the enzymatic preparation of pure AF which can be used as an attractive water soluble antioxidant 25 for food and non-food purposes. Examples are given below for the use of AF as an antioxidant in food formulations.

Experiments showed that the AF is comparable with known high quality commercial available food antioxidants.

30

Non-food examples include use in polymer chemistry as oxygen scavengers during the synthesis of polymers.

Also, AF could be used for the synthesis of bio-degradable plastic.

Experiments have shown that AF can be an efficient reducing agent (antioxidant), as it can easily reduce 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid.

5

AF is a naturally occurring substance and therefore it has a tremendous potential for use as an acceptable antioxidant. AF can also be converted into the antibiotic microthecin by AF dehydrase. Antibiotics are known for their uses in food biopreservation, an important area in food biotechnology.

10

In another aspect, the present invention also relates to the use of 1,5-D-anhydrofructose as a sweetener, in particular as a sweetener for foodstuffs and beverages, preferably human foodstuffs and beverages.

15

Thus according to this aspect of the present invention there is provided the use of 1,5-D-anhydrofructose as a sweetener.

Preferably the AF is used as or in a human foodstuff or beverage.

20

The AF may be used in any desired amount such as a 5% solution or 100mg/kg to 500 mg/kg.

25

The advantages of using AF as a sweetener are that it is a natural product, it is generally non-toxic, it is water soluble, it is likely that it is non-metabolisable at least for humans, and it is easy to manufacture.

The present invention therefore also relates to a novel application of AF as a sweetener.

30

Preferably the AF is prepared by the method according to the present invention.

Further aspects of the present invention include:

a method of preparing the enzyme α -1,4-glucan lyase (GL) comprising isolating the enzyme from algae alone;

5

a nucleotide sequence coding for the enzyme α -1,4-glucan lyase, preferably wherein the sequence is not in its natural environment (i.e. it does not form part of the natural genome of a cellular organism capable of expressing the enzyme, preferably wherein the nucleotide sequence is a DNA sequence;

10

the use of β -cyclodextrin to purify an enzyme, preferably GL.

Other preferred embodiments of the present invention include any one of the following: A transformed cell, tissue, organ or host organism having the capability

15

of producing AF as a consequence of the introduction of a DNA sequence as herein described; such a transformed host organism which is a microorganism - preferably wherein the host organism is selected from the group consisting of bacteria, moulds, fungi and yeast; preferably the cell, tissue, organ or host organism is obtained from or is any one of the group consisting of *Saccharomyces*, *Kluyveromyces*, *Aspergillus*,

20

Trichoderma Hansenula, *Pichia*, *Bacillus Streptomyces*, *Eschericia* such as *Aspergillus oryzae*, *Saccharomyces cerevisiae*, *bacillus sublilis*, *Bacillus amyloliquefascien*, *Eschericia coli*.; A method for preparing the sugar 1,5-D-anhydrofructose comprising the use of a transformed host organism expressing a nucleotide sequence encoding the enzyme α -1,4-glucan lyase, preferably wherein the nucleotide sequence is a DNA

25

sequence, preferably wherein the DNA sequence is one of the sequences hereinbefore described; A vector incorporating a nucleotide sequence as hereinbefore described, preferably wherein the vector is a replication vector, preferably wherein the vector is an expression vector containing the nucleotide sequence downstream from a promoter sequence, preferably the vector includes a marker (such as a resistance

30

marker); Cellular organisms, or cell line, transformed with such a vector; A method of producing the product α -1,4-glucan lyase or any nucleotide sequence or part thereof coding for same, which comprises culturing such an organism (or cells from

a cell line) transfected with such a vector and recovering the product.

In particular, in the expression systems, the enzyme should preferably be secreted to ease its purification. To do so the DNA encoding the mature enzyme is fused to a
5 signal sequence, a promoter and a terminator from the chosen host.

For expression in *Aspergillus niger* the gpdA (from the Glyceraldehyde-3-phosphate dehydrogenase gene of *Aspergillus nidulans*) promoter and signal sequence is fused to the 5' end of the DNA encoding the mature lyase. The terminator sequence from
10 the *A. niger* trpC gene is placed 3' to the gene (Punt, P.J. et al 1991 - (1991): J. Biotech. 17, 19-34). This construction is inserted into a vector containing a replication origin and selection origin for *E. coli* and a selection marker for *A. niger*. Examples of selection markers for *A. niger* are the amdS gene, the argB gene, the
15 pyrG gene, the hygB gene, the BmlR gene which all have been used for selection of transformants. This plasmid can be transformed into *A. niger* and the mature lyase can be recovered from the culture medium of the transformants. Eventually the construction could be transformed into a protease deficient strain to reduce the proteolytic degradation of the lyase in the culture medium (Archer D.B. et al 1992 - Biotechnol. Lett. 14, 357-362).

20

Instead of *Aspergillus niger* as a host, other industrial important microorganisms for which good expression systems are known could be used such as: *Aspergillus oryzae*, *Aspergillus* sp., *Trichoderma* sp., *Saccharomyces cerevisiae*, *Kluyveromyces* sp., *Hansenula* sp., *Pichia* sp., *Bacillus subtilis*, *B. amyloliquefaciens*, *Bacillus* sp.,
25 *Streptomyces* sp. or *E. coli*. Also, other forms of algae may be used.

The following sample was accepted as a deposit in accordance with the Budapest Treaty at the recognised depositary The Culture Collection of Algae and Protozoa (CCAP) at Dunstaffnage Marine Laboratory PO Box 3, Oban, Argyll, Scotland,
30 United Kingdom, PA34 4AD on 11 October 1994:

Gracilariopsis lemameiformis (CCAP 1373/2) - [ref. GLSC-1 (California)].

Thus a highly preferred embodiment of the present invention includes a GL enzyme or a nucleotide sequence coding for same obtainable from the algae that is the subject of deposit CCAP 1373/2.

- 5 The present invention will now be described only by way of example, in which reference shall be made to Figures which shows the results of some electrophoretic studies on algal α -1,4-glucan lyases, and Figure 2 which shows the results of some isoelectric focus studies on α -1,4-glucan lyases on a gel with a pH gradient of pH 3 to pH 9. Figures 1 and 2 are described in more detail later.

10

PREPARATION OF α -1,4-GLUCAN LYASE

The enzyme α -1,4-glucan lyase according to the present invention (e.g. for use in preparing AF) may be isolated from algae alone, preferably *Gracilaria* 15 *opis* *lemaneiformis*, more preferably *Gracilaria* *lemaneiformis* from Santa Cruz, (California).

The initial enzyme purification can be performed by the method as described by Yu *et al* (ibid). However, preferably, the initial enzyme purification includes an 20 optimized procedure in which a solid support is used that does not decompose under the purification step. This gel support further has the advantage that it is compatible with standard laboratory protein purification equipment. The details of this optimized purification strategy are given later on. The purification is terminated by known standard techniques for protein purification. The purity of the enzyme can be readily 25 established using complementary electrophoretic techniques.

CHARACTERISATION OF THE ENZYME

Amino acid sequence analysis

30

The α -1,4-glucan lyase from *Gracilaria* *lemaneiformis* was digested with either endoproteinase Arg-C from *Clostridium histolyticum* or endoproteinase Lys-C from

Lysobacter enzymogenes, both sequencing grade purchased from Boehringer Mannheim, Germany. For digestion with endoproteinase Arg-C, freeze dried lyase (0.1 mg) was dissolved in 50 μ l 10 M urea, 50 mM methylamine, 0.1 M Tris-HCl, pH 7.6. After overlay with N₂ and addition of 10 μ l of 50 mM DTT and 5 mM EDTA the protein was denatured and reduced for 10 min at 50°C under N₂. Subsequently, 1 μ g of endoproteinase Arg-C in 10 μ l of 50 mM Tris-HCl, pH 8.0 was added, N₂ was overlayed and the digestion was carried out for 6h at 37°C. For subsequent cysteine derivatization, 12.5 μ l 100 mM iodoacetamide was added and the solution was incubated for 15 min at RT in the dark under N₂.

10

For digestion with endoproteinase Lys-C, freeze dried lyase (0.1 mg) was dissolved in 50 μ l of 8 M urea, 0.4 M NH₄HCO₃, pH 8.4. After overlay with N₂ and addition of 5 μ l of 45 mM DTT, the protein was denatured and reduced for 15 min at 50°C under N₂. After cooling to RT, 5 μ l of 100 mM iodoacetamide was added for the cysteines to be derivatized for 15 min at RT in the dark under N₂.

15

Subsequently, 90 μ l of water and 5 μ g of endoproteinase Lys-C in 50 μ l of 50 mM tricine and 10 mM EDTA, pH 8.0, was added and the digestion was carried out for 24h at 37°C under N₂.

20

The resulting peptides were separated by reversed phase HPLC on a VYDAC C18 column (0.46 x 15 cm; 10 μ m; The Separations Group; California) using solvent A: 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides were rechromatographed on a Develosil C18 column (0.46 x 10 cm; 3 μ m; Dr. Ole Schou, Novo Nordisk, Denmark) using the same solvent system prior to sequencing on an Applied Biosystems 476A sequencer using pulsed-liquid fast cycles.

25

The amino acid sequence information from the enzyme derived from *Gracilaria* *lemaneiformis* is shown below (corresponds to SEQ. ID. No. 3) where the sequenced peptides are underlined.

30

	MFPTLTIFIAP	SALAASTFVG	ADIRSGIRIQ	SALPAVRNAV	RRSKHYNVSM	TALSDKOTAI
	<u>SIGPDNPDG</u> I	NYQNYDYIPV	AGFTP _L NSNT	WYAAGSSTPG	GITDWTATMN	VKFDRIDNPS
	YSNNHPVQIQ	VTSYNNNSFR	IRFNPDGPIR	DVSRGPILK <u>O</u>	LTWIRNOEL	AQGCNPMSF
	<u>SPEGFLSFET</u>	KDLNVIIYGN	CKMRVTKKDG	YLVMENDECN	SQSDGNKCRG	LMYVDRLYGN
5	AIASVQTNFH	KDTSRNEKFY	GAGEVNCRYE	EQGKAPTYVL	ERSGLAMTNY	NYDNLNYNQP
	<u>DVVPPGYPDH</u>	PNYYIPMYYA	APWLVVQGCA	GTSKQYSYGW	FMDNVSQSYM	NTGDTAWNCG
	QENLAYMGAQ	YGPFDQHFVY	GDGDGLEDDV	KAFSFLQGKE	FEDKKLNKRS	VMPPKYVFGF
	FQGVFGALSL	LKQNLPAGEN	NISVQEIVEG	YQDNDYPFEG	LAVDVDMQDD	LRVFTTKPEY
	WSANMVGEgg	DPNNRSVF EW	AHDRLVCQT	NVTCFLRNDN	SGKPYEVNQT	LREKQLYTKN
10	<u>DSLNNNTDFGT</u>	TSDGPGDAYI	GHLDYGGV E	CDAIFPDWGR	PDVAQWWGEN	YKKLFSIGLD
	<u>FVWQDMTVPA</u>	MMPHRLGDAV	NKNSGSSAPG	WPNENDPSNG	RYNWKS _Y HPO	VLVTDMRYGA
	EYGREPMVSQ	RNIHAYTLCE	STRREGIVGN	ADSLTKFRS	YIISRGGYIG	NQHFGGMWVG
	<u>DNSATESYLO</u>	MMLANIINMN	MSCLPLVGSD	IGGFTQYNDV	GDPTPEDLMV	RFVQAGCLLP
	WFRNHYDRWI	ESKKHGKKYQ	ELYMPGQKD	TLKKFVEFRY	RWQEVLYTAM	YQNATTGEPI
15	IKAAPMYNND	VNVYKSQNDH	FLLGGHDGYR	ILCAPVVREN	ATSREVYLPV	YSKWFKGPD
	<u>FDTKPLENEI</u>	OGGQTLYNYA	APLNDSPIFV	REGTILPTRY	TLDGVNKSIN	TYTDNDPLVF
	ELFPLENNQA	HGLFYHDDGG	VTTNAEDFGK	YSVISVKAAQ	EGSQMSVKFD	NEVYEHQWGA
	SFYVRVRNMG	APSINVSSQ	IGQQDMQQSS	VSSRAQMFTS	ANDGEYWVDQ	STNSLWLKLP
	GAVIQDAAIT VR					

In addition, for the characterization of α -1,4-glucan lyase from a red seaweed *Gracilariaopsis lemaneiformis* collected in Santa Cruz, California and apparently not infected by fungi, the following amino acid composition of the lyase was found:

	Amino acid residues	mol % of each residue

	Asx	15.42
	Thr	5.24
	Ser	6.85
10	Glx	9.46
	Pro	5.46
	Gly	9.08
	Ala	5.38
	1/2Cys	1.57
15	Val	6.60
	Met	2.90
	Ile	3.66
	Leu	6.00
	Tyr	6.00
20	Phe	4.37
	His	1.65
	Lys	4.44
	Arg	4.17
	Trp	1.75
25	Total:	100.00

Comparing the peptide sequences from the Californian algae with the amino acid sequence from a fungally infected algae from China (described in co-pending PCT patent application No. PCT/EP/0339) showed a high degree of homology (72-79% identity between the amino acid sequence in SEQ. ID. No. 3 and No. 4 generated from the PCR fragments and the corresponding sequences in the GL obtained from the algae from China) between the protein sequences.

The alignment of the protein sequences showed that sequence position 1 to 50 of SEQ. ID. No. 3 represents a signal sequence and sequence position 51 to 1092 represents the mature protein.

5 SEQ. I.D. No.3 has the following characteristics:

Number of residues:1092

Molecular weight (MW): 123169

Amino acid composition (including the signal sequence):

10

64	Ala	14	Cys	18	His	33	Met	
56	Thr	48	Arg	55	Gln	45	Ile	
49	Phe	22	Trp	89	Asn	49	Glu	
65	Leu	59	Pro	67	Tyr	73	Asp	
15	94	Gly	46	Lys	73	Ser	73	Val

SEQ. ID. No. 4 is a partial sequence corresponding to amino acid 287 to 861 in SEQ. ID. No. 3. The identity between the two sequences is 81%.

20

DNA sequence analysis

25

DNA from *Gracilariaopsis lemaneiformis* was isolated as described by Saunders (1993) with the following modification: The polysaccharides were removed from the DNA by ELUTIP-d (Schleicher & Schuell) purification instead of gel purification. (Ref: Saunders, G.W. (1993). Gel purification of red algal genomic DNA: An inexpensive and rapid method for the isolation of PCR-friendly DNA. Journal of phycology 29(2): 251-254 and Schleicher & Schuell: ELUTIP-d. Rapid Method for Purification and Concentration of DNA.)

30

For PCR, the appropriate DNA molecule was prepared by use of the Gene Amp DNA Amplification Kit (Perkin Elmer Cetus, USA) and in accordance with the manufacturers instructions except that the Taq polymerase was added later (see PCR cycles) and the temperature cycling was changed to the following:

5

PCR cycles:

	no of cycles	C	time (min.)
10	1	98	5
		60	5
addition of Taq polymerase and oil			
15	35	94	1
		47	2
		72	3
20	1	72	20

20

PCR fragments were cloned into pT7Blue (from Novagen) or ScriptTMSK(+) following the instructions of the supplier.

The double stranded DNA was sequenced essentially according to the dideoxy method
25 of Sanger *et al.* (1979) using the Auto Read Sequencing Kit (Pharmacia) and the
Pharmacia LKB A.L.F.DNA sequencer. (Ref: Sanger, F., Nicklen, S. and Coulson,
A.R.(1979). DNA sequencing with chain-determinating inhibitors. Proc. Natl. Acad.
Sci. USA 74: 5463-5467.). The sequences are shown as SEQ. ID. No.s 1-2.

30 For the initial PCR, the following three oligonucleotide sequences were generated from two peptide sequences from the Californian algae to generate a PCR fragment of app. 970 bp.

35

Primer 1: ATGAC(GATC)AA(CT)TA(CT)AA(CT)TA(CT)GA(CT)AA

Primer 2: (AG)TG(GATC)GGCATCAT(GATC)GC(GATC)GG(GATC)AC

5 Primer 3: GTCAT(GA)TC(CT)TGCCA(GATC)AC(GA)AA(GA)TC

Primer 1 corresponds to the codons for amino acids 287-293 in SEQ. I.D. No. 3.

10 Primer 2 and primer 3 correspond to codons (of the complementary strands) for amino acids 608-614 and 599-607 of SEQ. ID. No. 3, respectively.

15 Primer 1 was used as the upstream primer and primer 2 was used as the downstream primer in the first PCR amplification. In the second PCR amplification primer 1 was used as the upstream primer and primer 3 was used as the downstream primer. A PCR fragment of the expected size was generated and cloned into the pT7blue vector from Novagen. Two independent plasmids containing a PCR fragment were sequenced and it was seen that those two cloned PCR fragments contained the codons for peptide sequences originating from two different proteins. This indicates that there are at least two different genes coding for α -1,4-glucan lyase in the Californian 20 algae containing sequences shown as SEQ. ID. No.s 1-2 - their putative amino acid sequences are shown as SEQ. ID. No.s 3-4.

25 Further PCR using the Pfu polymerase from Stratagene were made according to the manufacturers instruction using the same temperature cycling as for the Taq polymerase. The PCR fragments were cloned into either the pT7Blue plasmid from Novagen or the pCR-ScriptTM SK(+) plasmid from Stratagene.

Four additional PCR amplifications were performed using the following primers.

For the first PCR the primer

GA(CT) AA(CT) CC(GATC) GA(CT) GG(GATC) AT(ATC) (GA)A(CT) TA

5

corresponding to codons for amino acids 65-72 of SEQ. ID. No.3 was used as the upstream primer and the primer

10

(GA)GA (TG)AC ATT (GA)TC CA(AT) AAA CCA

generated from the initial PCR corresponding to codons (of the complementary strand) for amino acids 340-346 of SEQ. ID. No.3 was used as the downstream primer.

In the second PCR the primer

20

GT(GA) GAT GT(GT) GAT ATG CAA (GC)A(AT) GA

generated from the initial PCR corresponding to codons for amino acids 463-470 of SEQ. ID. No.3 was used as the upstream primer and the primer

CCA CAT (GATC)CC (GATC)CC (GA)AA (GA)TG (CT)TG (GA)TT

30

corresponding to codons (of the complementary strand) for amino acids 711-718 of SEQ. ID. No.3 was used as the downstream primer.

In the third PCR the primer

G TG AGT CTA CTA GGA GGG AA

- 5 generated from the initial PCR corresponding to codons for amino acids 389-395 of SEQ. I.D.No.4 was used as the upstream primer and the primer

A(GA) (GA)AA (GA)TG (GA)TC (GA)TT (CT)TG

- 10 corresponding to codons (of the complementary strand) for amino acids 566-571 of SEQ. ID. No. 4 was used as the downstream primer.

In the fourth PCR the primer

- 15 TTC CCA GA(CT) TGG GGT CGA CC

generated from the initial PCR corresponding to codons for amino acids 575-581 of SEQ. ID. No. 3 was used as the upstream primer and the primer

- 20 GT(GA) AA(GA) TC(GATC) GG(GATC) CC(GA) AA

corresponding to codons (of the complementary strand) for amino acids 897-902 of SEQ. ID. No. 3 was used as the downstream primer.

- 25 The first PCR amplification generated a DNA sequence corresponding to nucleotides 193-1038 in SEQ. ID. No. 1.

The second PCR amplification generated DNA sequences corresponding to nucleotides 1387-2154 in SEQ. ID. No. 1 and nucleotides 526-1284 in SEQ. ID. No.

- 30 2.

The third PCR amplification generated a DNA sequence corresponding to nucleotides 1165-1712 in SEQ. ID. No. 2.

- 5 The fourth PCR amplification generated a DNA sequence corresponding to nucleotides 1723-2706 in SEQ. ID. No. 1.

To obtain the 5' and 3' end of SEQ. ID. No.3 the RACE (rapid amplification of cDNA ends) procedure was performed (Michael, A.F., Michael, K.D. & Martin, G.R.(1988). Proc.Natl.Acad.Sci.USA 85:8998-9002) using the 5'Race system from Gibco BRL for the 5'Race. Total RNA was isolated according to Collinge *et al.*(Collinge, D.B., Milligan, D.E., Dow, J.M., Scofield, G. & Daniels, M.J.(1987). Plant Mol Biol 8:405-414). The 5'Race was done according to the protocol of the manufacturer, using 1 μ g of total RNA and the sequence

GGT AGC GGT CCA GTC GGT GAT GCC

20 (identical to the complementary strand of nucleotides 301-324 in SEQ. ID. No. 1) as a primer in the first strand synthesis.

The sequence

25 AGA GCC GGC AGC ATA CCA GTT GGT GTT GG

identical to the complementary strand of nucleotides 260-288 in SEQ. ID. No. 1 was used for the PCR amplification.

In the 3'Race the following primer

5

GAA GGA TCC GTC GAC ATC GAT AAT ACG ACT GAA TTC GGG ATT TTT
TTT TTT TTT TTT

was used for the first strand synthesis.

10

The gene specific primer

GAC GGC TAT CGT ATT CTC TGC

15 identical to nucleotides 2599-2619 in SEQ. ID. No. 1 was used in the first PCR amplification, while primer

TAC CTG CCT GTG TAT AGC AAG

20 corresponding to nucleotides 2659-2679 was used as the gene specific primer in the second PCR amplification.

The sequence

25 AAG GAT CCG TCG ACA TCG ATA AT

was used as the downstream primer in both PCR amplifications. The GeneAmp RNA PCR kit from Perkin Elmer was used for the 3'Race.

30 After the 5' and 3' Race the PCR fragments were blunt ended with the Pfu polishing kit from Stratagene before cloning into the pCR ScriptTM SK(+) plasmid.

BIOCHEMICAL CHARACTERIZATION

Electrophoretic properties of the α -1,4-glucan lyase purified from the red alga *Gracilaria* collected in Santa Cruz, Californian (USA), as compared with 5 lyases collected in Qingdao (China) and Sucre Peninsula (Venezuela), and with lyases isolated from the fungi *Morchella costata*, *M. vulgaris*.

Two lyases (GLsc1-lyase and GLsc2-lyase) were purified from the red alga *Gracilaria* collected in Santa Cruz, California (USA), using the 10 procedure developed for the purification of the lyase from the red alga collected from Qingdao China. The two forms were purified to electrophoretic homogeneity (Figure 1A and 1B). Partial amino acid sequences were obtained from the purified GLsc1-lyase and were used to generate PCR primers. The molecular mass was estimated to 116 kDa for both GLsc1-lyase and GLsc2-lyase, by the SDS-PAGE method (Figure 15 1A).

On the native PAGE (Figure 1B), GLsc1-lyase and GLsc2-lyase migrated slower than GL1-lyase. On the isoelectrofusing gel, GLsc1-lyase showed a *pI* of 4.1 (Fig. 2). A comparison of the relative migration rates and the *pI* values of the different lyases 20 studied are listed in the following table.

Enzyme name	Origin	Migration	<i>pI</i>
GL1-lyase	QC	++++	3.8
GLsc1-lyase	SCC	+++	4.1
GLsc2-lyase	SCC	+++	4.1
GLv-lyase	SpV	+++	4.1
MV-lyase	MvA	++	4.4
MC-lyase	MCA	+	4.5

CODES:

QC = Qingdao, China

SCC = Santa Cruz, California

SpV = Sucre peninsula, Venezuela

5 MvA = *Morchella vulgaris*, ATCC

McA = *Morchella costata*, ATCC

The relative migration rates were estimated from native PAGE on gels with 8-25% gradient; the *pI* values were estimated by isoelectrofocusing on gels with a pH 10 gradient of 3-9. From the table it is seen that the GLq1-lyase exhibited the fastest migration rate on native-PAGE and exhibited the lowest *pI* values of 3.8 while the MC-lyase showed the slowest migration rate and the highest *pI* value of 4.5.

The Figures are now discussed in more detail.

15

Figure 1. Electrophoresis of algal α -1,4-glucan lyases.

A. SDS-PAGE on 8-25% gradient gels. The gel was stained with PhastGel Blue R.

20 Lane 1: the molecular markers of phosphorylase b (97.4kDa); bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and β -lactalbumin (14.4 kDa).

25 Lane 2: the purified GLq1-lyase from the red alga *Gracilariaopsis lemaneiformis* collected in Qingdao (China).

Lanes 3 and 5: the purified GLsc1-lyase from the red alga *Gracilariaopsis lemaneiformis* collected in Santa Cruz, California (USA).

30 Lane 4: the purified GLsc2-lyase from the red alga *Gracilariaopsis lemaneiformis* collected in Santa Cruz, California (USA).

B. Native-PAGE on 8-25% gradient gel. The gel was stained with PhastGel Blue R.

Lane 1 and 4: the purified GLq1-lyase from the red alga *Gracilariaopsis lemameiformis* collected in Qingdao (China).

5

Lane 2: the purified GLsc2-lyase from the red alga *Gracilariaopsis lemameiformis* collected in Santa Cruz, California (USA).

10 Lane 3: the purified GLsc1-lyase from the red alga *Gracilariaopsis lemameiformis* collected in Santa Cruz, California (USA).

Lane 5: the protein markers: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa).

15 **Figure 2.** Isoelectric focusing of the α -1,4-glucan lyases on a gel with a pH gradient of pH 3 to pH 9. The gel was stained with PhastGel Blue R.

Lane 1: GLsc1-lyase purified from *Gracilariaopsis lemameiformis* collected in Santa Cruz California showing a pI of 4.1.

20

Lane 2: GLq1-lyase purified from *G. lemameiformis* collected in Qingdao (China) showing a pI of 3.8.

25 Lane 3: the pI markers: 1 amyloglucosidase (3.50), 2 soybean trypsin inhibitor (4.55), 3 β -lactoglobulin A (5.20), 4 bovine carbonic anhydrase B (5.85), 5 human carbonic anhydrase B (6.55), 6 horse myoglobin (acidic, 6.85), 7 horse myoglobin (basic 7.35), 8 lentil lectin (acidic 8.15), 9 lentil lectin (middle, 8.45), 10 lentil lectin (basic, 8.65).

30 Further studies showed that K_m was 3.76 mg/ml for amylopectin and 3.37 mg/ml for glycogen.

DEGRADATION STUDIES

The degradation rates of the lyase on various substrates are given below.

	Substrate	AF released (nmol)
5	Maltose	657
10	Maltotriose	654
15	Maltotetraose	670
20	Maltopentaose	674
25	Maltohexaose	826
	Maltoheptaose	865
30	Dextrin 20	775
35	Dextrin 15	775
40	Dextrin 10	844
45	Amylopectin	732
50	Glycogen	592

30 Reaction conditions: The reaction mixture contained 10 mM of HOAc-NaOAc (pH 3.8). The substrate concentration was 10 mg/ml. The final volume was 100 ul after the addition of lyase and water. The reaction time was 40 min at 45°C.

35 The lyase was not able to degrade pullulan, nigeran tetrasaccharide, trehalose, isomaltose, glucose, α -, β - and γ -cyclodextrins. The lyase degraded panose and nigerose though at a slow rate.

40 The temperature optimum for the lyase was 48°C when amylopectin was used as substrate and 50°C when glycogen was used as substrate. At 50°C, the reactivity of glycogen was similar to that of amylopectin; below 50°C, amylopectin was a better substrate than glycogen.

The pH optimum range for the lyase was between pH 3.5 and pH 7.0; the optimal pH was 3.8. The buffers used in the pH tests were glycine-HCl (pH 2.2-3.6); NaOAc-HOAc (pH 3.5-5.5); Mes-NaOH (pH 5.5-6.7); Mops-NaOH (pH 6.0-8.0) and bicine-NaOH (pH 7.6-9.0). All buffers used were 40 mM.

5

At a final concentration of 2 mM, p-chloromercuribenzoic acid (PCMB) inhibited the lyase activity by 96%, indicating the -SH group(s) is essential for the enzymatic activity.

- 10 In immunological tests of the lyase by Western blotting, the results showed that the antibodies to the algal lyase could recognize the fungal lyase (of our earlier PCT patent application PCT/EP94/03397) both in cell-free extracts and in purified form, as revealed by Western blottings. The antibodies to the algal lyase purified form the algae collected in China (of our earlier PCT patent application PCT/EP94/03397) also
15 recognized the lyase from the algae collected in Santa Cruz, California.

Further aspects and experiments relating to the enzyme according to the present invention will now be described.

20 THE SOLUBLE ENZYME SYSTEM

1. Effect of pH on the stability and activity of the lyase isolated from *Gracilariaopsis lemaneiformis*.
- 25 Two buffer systems, namely HOAc and NaOAc and sodium citrate - citric acid in a concentration of 5 mM - were tested at 37°C. The pH range tested was from pH 3 to pH 5.2. The lyase showed maximum activity in a pH range between 3.6 to 4.2. At pH 3, the stability and activity of the enzyme decreased by about 90%. At pH 5.2, the activity decreased by about 64%. However, the enzyme was considerably more stable at this pH than at pH 3, as the AF yield obtained at pH 5.2 was 75% of the AF
30 yield obtained at pH 3.8. Slightly higher AF yield was obtained in the HOAc and NaOAc buffer than in citrate buffer. This is not due to any differential effect of the

two buffers (final conc. is 125 μM in the AF assay mixture) in the AF assay method.

2. Effect of temperature on the activity and stability of the lyase.

- 5 This experiment was conducted at optimal pH range. At 25°C the production of AF was linear up to at least 9 days. This indicates that no loss of activity and stability of the lyase occurred within 9 days. With increasing temperature, the stability of the enzyme decreased.
- 10 The half life of the enzyme activity at the following temperature was:
- | | |
|---------|-----------------|
| 30°C | 5 days |
| 37°C | 2.5 days |
| 40°C | less than 1 day |
| 15 50°C | less than 1 day |

3. Effect of substrate concentration on the stability of the lyase and AF yield.

- 20 It was observed that amylopectin and dextrins have a stabilizing effect on the lyase while the smallest substrate maltose does not. This was verified for both the soluble enzyme system and the immobilized enzyme system.

- 25 AF yield increases with the increase in amylopectin concentration up to 25%. In the case of dextrin, the AF yield decreases when the concentration exceeds 30% (30%, 40% and 50% were tested).

4. Activation and inactivation of lyase

- 30 No metal ions were found to be necessary for the activity and the enzyme catalysed reaction can surprisingly proceed in pure water. The fact that the addition of EDTA in the reaction mixture up to 20 mM had little effect on the activity clearly demonstrates that metal ions are not essential for the activity of the lyase enzyme

according to the present invention.

This means that in the AF purification step, the ion exchange chromatography step that takes away salts from the reaction system can be omitted, if water is used as reaction medium. However, inclusion of NaCl in the reaction mixture in a concentration of 0.85% (0.145 M) can increase the AF yield up to 1-fold.

5. Substrate Specificity

10 Upon cooling solubilized starch will tend to form rigid gels when the starch concentration becomes too high. Therefore it is an advantage to utilize partly degraded starch as substrate for the 1,4-glucan lyase.

15 The specificity of α -1,4-glucan lyase for different oligosaccharides was tested. The oligosaccharides were maltose (G2), maltotriose (G3), maltotetraose (G4), malto-pentaose (G5), maltohexaose (G6) and maltoheptaose (G7). The oligosaccharides were dissolved in H₂O at a concentration of 8 mg/ml. The enzyme assay contained 150 μ l substrate G2/G3/G4/G5/G6/G7, 120 μ l 0.1M MES pH 6.3 and 30 μ l purified enzyme. The reaction mixture was incubated for 60 min at 30°C. Afterwards the 20 reaction was stopped by boiling for 3 min and 900 μ l absolute ethanol was added for precipitation. After centrifugation at 20.000 x g for 5 min at 4°C the supernatant was transferred to a new eppendorf tube and lyophilized.

25 The freeze-dried samples were dissolved in 1000 μ l H₂O and were filtrated through a 0.22 μ m Millipore filter before 25 μ l of the sample was loaded on the Dionex HPLC.

6. HPLC

30 Analyses were performed on a Dionex 4500i chromatography system consisting of a GPM-2 pump and a PED detector which was used in pulse-amperometric detection mode. The anion exchange columns were a CarboPac PA-100 (4 x 250 mm) and a

CarboPac PA-100 guard column (3 x 25 mm) from Dionex. The eluent were 200 mM sodium hydroxide (A), 500 mM sodium acetate (B) and 18 M ohm de-ionized water (C). The pump was programmed in 2 different ways, method no. 1 and method no. 2:

5

Method no. 1:

Time, min.	0.0	3.0	3.1	26.9	29.0
% A	10	10	50	50	10
% B	0	0	0	32	0
10 % C	90	90	50	18	90

Method no. 2:

Time, min.	0.0	30
% A	10	10
% B	0	0
% C	90	90

Standards:

20 Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose (all from Sigma) and 1,5-anhydrofructose were used as standards. All compounds were dissolved in 18 M ohm de-ionized water which was filtered through a 0.22 μm Millipore filter before use.

7. Results

25

The analyses show that the purified enzyme was able to use maltooligosaccharides as substrate 1 for 1,5-anhydrofructose formation. When maltose was used as substrate, almost no 1,5-anhydrofructose was formed but when the other maltooligosaccharides

(G3-G7) were used, high amounts of this compound were produced. It is clear that higher amounts of 1,5-anhydrofructose were obtained when a longer maltooligosaccharide was used. This observation corresponds perfectly well with the theory of the lyase forming 1,5-anhydrofructose from the non-reducing end of the substrate,
5 leaving only the terminal glucose molecule unchanged.

8. Formation of AF

10 α -1,4-glucan lyase according to the present invention hydrolyses starch to the end-product 1,5-anhydrofructose. The end-product was shown by HPLC, method 2. The enzyme assay contained 500 μ l amylopectin (20 mg/ml, dissolved in H₂O), 400 μ l 0.1 M MES pH 6.3 and 100 μ l purified enzyme. The reaction mixture was incubated at 30°C and the reaction was stopped by boiling after 30 or 120 min incubation. High-molecular oligosaccharides were precipitated by addition of 3 vol abs. ethanol and the
15 sample was centrifuged and freeze-dried as described above. The samples were dissolved in 125 μ l H₂O and 25 μ l were applied on the HPLC column.

20 The HPLC elution profile clearly shows that α -1,4-glucan lyase produces 1,5-anhydrofructose by hydrolysis of starch. Equal amounts of 1,5-anhydrofructose were found after 30 and 120 min. incubation which indicate that the enzyme activity is not inhibited by the end-product 1,5-anhydrofructose.

25 ¹³C NMR spectra (water) of AF prepared in this way shows that it adopts one major form giving rise to the following signals: δ 93.5 (quart, C-2), 81.5 (CH, C-5), 77.7 (CH, C-3), 72.6 (CH₂, C-1), 69.8 (CH, C-4), 62.0 (CH₂, C-6). Assignments are based on H-H C-H and C-H 2D correlation spectra.

9. The cooperative effect of lyase with pullulanase and isoamylase.

30 As it can be seen from the Table below, the inclusion of pullulanase in the reaction mixture will obviously increase the AF yield by about 15-23%, depending on whether soluble starch or amylopectin is used as substrate.

The cooperation of pullulanase and lyase in the production of AF.

Substrate	Lyase	Pullulanase	AF Yield (%)	Glc Yield (%)
<hr/>				
Solubl.				
Starch	+	-	51	0
	-	+	0	0.37
<hr/>				
	+	+	66.0	3.9
<hr/>				
Amylo	+	-	48.0	0
-pectin				
	-	+	0	0.33
<hr/>				
	+	+	71.3	3.7
<hr/>				

+, enzyme added, - enzyme omitted.

The reaction mixture contained 0.3 ml 2% potato amylopectin (Sigma) in water or 0.3 ml 2% soluble starch (Merck), 2 μ l lyase and 0.36 units pullulanase (BM) as indicated.

5

The reaction was carried out at 30°C for 1 day. At the end of the reaction, samples were taken for AF and Glc analysis.

In the case of isoamylase, the advantage is that the optimal pH of the lyase overlaps with that of Pseudomonas isoamylase (pH 3.0-4.5). The problem, however, is that isoamylase will produce an excess amount of long chain amylose that precipitates from the solution, and therefore is no longer suitable as a substrate for the lyase. It can be expected that the cooperation of the lyase with isoamylase will be efficient, if the chain of amylose is not too long.

10

15

THE IMMOBILIZED ENZYME SYSTEM

Immobilization of the lyase was achieved by using succinimide-activated Sepharose (Affigel 15 gel, Bio-Rad) and glutaraldehyde-activated Silica gel (BM). The recovery of lyase activity after immobilization on Affigel 15 gel was between 40% to 50%.
5 There may be some lyase that is still active after immobilization, but is inaccessible to the substrate because of the steric hindrance, especially in the case of macromolecules like starches. Immobilized enzymes used in the industry usually have an activity recovery of around 50%.

10

The most interesting thing of the Affigel 15 gel immobilized lyase is that its stability has been greatly improved at pH 5.5. When the column was operated at this pH, the stability was at least 16 days long. The pH shift in the stability is very important considering the optimal pH of pullulanase which is around pH 5.5. This is the prerequisite for the lyase and pullulanase to cooperate efficiently in the same reactor with the same physico-chemical environment. The soluble lyase has an optimal pH between 3.6 and 4.2, and at this pH range pullulanase shows little or no activity.
15

With the silica gel immobilized lyase, the activity recovery is very high, around 80-100%. However, the silica gel immobilized enzyme was not stable when the column was operated neither at pH 3.8 nor pH 5.5. It is possible that some lyase was adsorbed on the surface of the silica gel beads and was slowly released from the silica gel after each washing of the column. It may therefore be the adsorbed lyase that contributes to the high recovery rate and the decrease in column activity.
20

25

PURIFICATION OF AF

1. The lyase-Amylopectin/Soluble Starch System

In this system, the reaction system contained AF, limit dextrin, the lyase, and buffer salts at the end of the reaction. AF was separated from the macromolecules (limit dextrin and the lyase) by ethanol (final conc. 50%) precipitation. Unprecipitated low-

molecular-weight amylopectin was separated by ultrafiltration using Amicon YM3 membranes (cut-off 3,000). Ethanol was removed by evaporation at 40°C in a rotary evaporator. Buffer salts were removed from AF by mixed ion exchangers. Purified solid AF was obtained by freeze-drying.

5

2. The Lyase-Pullulanase/Amylopectin/Soluble Starch System.

10

In this system the final products are AF and glucose. If at least a substantially pure sample of AF is to be prepared, the by-product glucose must be removed. This can be achieved by enzymatic methods. First the glucose is converted into gluconic acid and hydrogen peroxide by glucose oxidase.

15

Catalase is needed to dispel H₂O₂ formed. H₂O₂ will oxidize AF into two new compounds which are at present of unknown structure. The other impurities in the AF preparation are the oxidation products of AF. It was observed that AF can slowly be oxidized by air-level of oxygen, especially at high temperature, high AF concentration and long time of exposure. Gluconic acid was removed together with the buffer salts by ion exchange chromatography.

20

In this system, the low-molecular-weight amylopectin molecules may alternatively be hydrolysed by amyloglucosidase instead of using ultrafiltration.

3. The purity checking of AF.

25

The purity of the AF preparations were confirmed by TLC, Dionex and NMR.

4. Analysis of the antioxidative activity of anhydro fructose.

Electrochemical oxygen consumption:

30

The activity of AF was investigated in a methyl linoleate emulsion as described by Jorgensen and Skibsted (Z. Lebensm. Unters. Forsch. (1993) 196: 423-429) with

minor modifications: To 5.00 ml of a 1.33 mM methyl linoleate emulsion in 5.0 mM aqueous phosphate buffer with pH = 5.8 and 0.2 w/w % Tween 20 as emulsifier was added AF in the following concentrations: 0, 15, 146 and 680 μ M. The oxidation in the system was initiated by addition of 50 μ l 0.26 M metmyoglobin (MMb) final concentration 0.26 mM. Immediately after initiating the reaction the sample was injected to a thermostated ($25.0 \pm 0.1^\circ\text{C}$) 70 μ l closed cell, effectively excluding diffusion of oxygen into the system. The oxygen consumption was measured by a Clark electrode, which was connected to a PC data collection program. The relative oxygen concentration (%) was registered every 30s.

10

The results of the experiments produced curves corresponding to oxygen consumption. For samples without addition of AF a relative decrease in oxygen concentration is seen immediately after injection of the sample. For samples containing AF a lag-phase is observed before the curve breaks off and the oxygen concentration is reduced. After the lag-phase only a minor reduction in the oxygen consumption rate is observed compared to samples without AF added. A tendency for samples having the highest amount of AF to have the longest lag-phase is observed. Also, the rate for oxygen consumption is lower for these samples, which was seen by the smaller slope of the curves compared to the slope for the references (0 μ M).

15

ESR analysis:

Hydroxyl radicals were generated by a Fenton reaction with H_2O_2 (0.17 mM) and FeSO₄ (4.8 μ M). The generated radicals were trapped by 5,5-dimethyl-1-pyrroline N-oxide (DMPO, 9.7 mM). AF was added in concentrations of 1.3 mM and 6.3 mM. A water soluble extract of rosemary (*Rosmarinus officinalis* L.) was analyzed in a concentration of 0.25 mg/ml (in grams equivalent to 1.26 mM AF). Measurements were carried out at room temperature ($20 \pm 1^\circ\text{C}$) after 120 s and repeated for the same reaction mixture after 300 s with the following spectrometer settings: Center field 3475.60 G; sweep width 55 G; microwave power 20 mW; modulation frequency 100 kHz; modulation amplitude 1.01 G; receiver gain $1.00 \cdot 10^5$; conversion time 81.92 ms time constant 163.84 ms and sweep time 83.89 s.

The results showed that the generated hydroxyl radicals were trapped by DMPO. The spin adduct gives rise to a characteristic 1:2:2:1 ESR spectrum. The peak height of the spectrum is proportional to the quantitative amount of generated spin adduct. Addition of both DMPO and AF will set up a competition between the spin trap and AF. A reduction of peak height will indicate a good scavenging activity of AF.

Table: Peak height of ESR-spectra. $H_2O_2 = 0.17\text{mM}$ and $Fe^{2+} = 4.8 \mu\text{M}$.

Anhydro fructose [mM]	Rosemary extract [mg/ml]	Peak height [120 s]	Peak height [300 s]
0	0	2475	2780
1.3	0	2634	2545
6.3	0	1781	1900

At a concentration of 1.3 mM AF no scavenging activity of hydroxyl radicals is seen, at 6.3 mM AF the peak height is reduced, indicating that a part of the generated hydroxyl radicals is scavenged by AF.

USE OF AF AS AN ANTI-OXIDANT

1. Use of AF as an anti-oxidant in a 50% mayonnaise.

50% mayonnaise is used for salads, open sandwiches, etc. in both the catering and the retail trades. The low oil content of 50% mayonnaise makes it suitable for low-calorie applications.

A typical mayonnaise composition is as follows:

	Soya oil	50.0%
	Tarragon vinegar (10%)	4.0%
5	Egg yolk	3.5%
	Sugar	3.0%
	Salt	1.0%
	Potassium sorbate	0.1%
	Water	35.2%
10	MAYODAN 602	3.0%
	Lemon flavouring 10251	0.2%

MAYODAN 602 ensures a fine, stable oil dispersion and the required viscosity, thereby providing 50% mayonnaise with a long shelf life.

- 15 Flavouring 10251 is a natural lemon flavouring which provides mayonnaise with the fresh taste of lemon.

Typically the mayonnaise is prepared by the following method:

- 20 1) Dry mix the MAYODAN 602, sugar and salt. Disperse in oil in a ratio of 1 part powder to 2 parts oil.
- 25 2) Add flavouring and potassium sorbate to the water and pour into the Koruma mixer. Add 1).
- 3) Add the egg yolk.
- 4) Add the oil continuously in a vacuum.
- 30 5) After 2/3 of the oil has been added (slowly), blend the tarragon vinegar with the remaining 1/3 of the oil, and add.

The following data show that when AF is added to the mayonnaise as an anti-oxidant the results are comparable to the known food anti-oxidants GRINDOX 142 and GRINDOX 1029.

5 GRINDOX 142:

	Ascorbyl palmitate	10%
	Propyl gallate	20%
	Citric acid	10%
	Food grade emulsifier	60%
10	Form at 25°C	paste
	Colour	grey to pale brown
	Density	1.1 g/ml

(All percentages are by weight)

15 GRINDOX 1029:

	Ascorbyl palmitate	20%
	Natural tocopherols	20%
	Food grade emulsifier	60%
	Form at 25°C	paste
20	Colour	light brown
	Density at 25°C	1.0 g/ml

(All percentages are by weight)

25 In the test procedure the anti-oxidants were added to the mayonnaise to provide an anti-oxidant concentration in the order of about 500 ppm. The mayonnaise was then placed in a bomb calorimeter at temperature 80°C containing pure O₂. An induction period to the onset of substantial oxidation of the product is then measured.

30 The results were as follows.

Samples:		<u>IP (hours)</u>
1.	Blank	28,0
2.	+ 500 ppm GRINDOX 142	35,0
3.	+ 500 ppm GRINDOX 1029	33,3
5	4. + 550 ppm GRINDOX 1029	34,3
	5. + 500ppm 1,5 anhydro-D-fructose	32,0

(IP hours = Induction Period)

These results show that AF is an excellent food anti-oxidant and is comparable with
10 the known foodstuffs anti-oxidants GRINDOX 142 or GRINDOX 1029.

2. Use of AF as an anti-oxidant in a salad dressing

YOGURT SALAD DRESSING WITH 50% OIL

15 Yogurt salad dressing with 50% oil is used for salads, potatoes, raw vegetable salad, meat, fish and boiled vegetables.

Composition

20	Soya oil	50.0%
	Yogurt (plain)	39.0%
	Vinegar (10%)	3.5%
	Sugar	3.0%
	Egg yolk	2.0%
25	Salt	1.0%
	Potassium sorbate	0.1%
	MAYODAN 525	1.4%
	Acid masking flavouring 2072	0.02%

30 MAYODAN 525 provides unique emulsion stability, prevents syneresis, ensures uniform oil dispersion and viscosity, improves tolerance to production processes and ensures a long shelf life.

Flavouring 2072 is a nature-identical, acid masking flavouring reducing the acidulated taste of dressing without affecting its pH value.

Method

- 5 1. Dry mix MAYODAN 525, sugar and salt. Disperse in oil in a ratio of 1 part powder to 2 parts oil.
2. Fill flavouring, potassium sorbate and yogurt into the Koruma mixer. Add 1).
3. Add the egg yolk.
- 10 4. Add the oil continuously in a vacuum.
5. After 2/3 of the oil has been added (slowly), blend the vinegar with the remaining 1/3 of the oil, and add.
6. Add spices if required.

15 Test results:

Sample:	<u>IP hours</u>	<u>PF</u>
1. Blank	37.2	1.00
2. 500 ppm anhydrofructose	39.5	1.06
20 3. 800 ppm GRINDOX 1032	43.3	1.07

(IP - Induction Period); (PF - Protection Period)

Protection Factor (PF):

For each temperature defined as

25 PF = IP of the oil with added antioxidant/IP of the same oil without added antioxidant

Life extension (LE) %:

$$\text{LE} = (\text{PF} - 1.0) \times 100$$

FURTHER STUDIES

1. Effect of alcohols in increasing the activity and stability of the lyase purified from the algae.

5

1-propanol, 2-propanol and 1-butanol were tested at the following concentrations (0%, 1%, 5% and 10%). The optimal concentration of 1-propanol was 5% which increased the AF yield by 34% after 6 days of incubation; the optimal concentration for 2-propanol was 1% which increased the AF yield by 20% after 10 days incubation; the optimal concentration for 1-butanol was 5% which increased the AF yield by 52% after 3-day incubation.

10

Ethanol was tested at the following concentrations (0, 1, 3, 5, 7, 9, 11, 13, 15%). The optimal concentration for 7 days incubation was 5% which increased the AF yield by 12%. For 10 days incubation the optimal concentration was 3% which increased AF yield by 16%.

15

The effect of 1-propanol:

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	1-propanol concentration (v/v)	Reaction time (days)				
		0	1	3	6	10
		AF yield (μ mol)				
	0%	0	84	261	451	689
25	1%	0	80	280	530	803
	5%	0	115	367	605	853
	10%	0	107	307	456	583

30

2. Effect of different reaction media upon the production of AF by the lyase purified from the algae

The results (see table below) indicate that the best reaction medium is 5 mM of HOAc-NaOAc (pH 3.9) (BACE for short) and containing mM concentrations of Na₂-EDTA. The production of AF using either pure water or 0.85% NaCl as reaction medium decreased the yield. Inclusion of 0.85% of NaCl in BACE also decreased the AF yield.

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Reaction Media	Reaction Time (days)			
	0	1	3	8
AF yield (μ mol)				
BACE	0	229	498	575
Water	0	46	128	217
NaCl (0.85%)	0	123	239	249
BACE+NaCl (0.85%)	0	153	281	303

The following buffers: Mes-NaOH, Mops-NaOH, Hepes-NaOH, and Bicine-NaOH were the optimal reaction media for the lyase.

3. The effect of endoamylases and debranching enzymes upon the AF production.

Endoamylase

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The starch used for AF production may first be liquified either by endoamylases, or by acid hydrolysis.

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Endoamylase degraded starch is more suitable as substrate for the lyase as compared to native starch. Starch has a limited solubility at the temperature used for the lyase-catalyzed reaction. Treatment of starch with endoamylases led to increased glucose yield. It was found that a reducing matter of around 10-15% (on a dry mater basis) was most suitable as substrate for the lyase with respect to AF yield and further

treatment with the endoamylase to a reducing matter of 19% was no longer suitable for the lyase.

Pullulanase and isoamylase

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As seen from the results below, both the isoamylase and the pullulanase increased AF yield by up to 50% at pH 4.5 and 5.0. The reaction system consisted of the lyase from the algae with or without the addition of isoamylase or pullulanase (MegaZyme Ltd.). Amylopectin was used as substrate. The AF produced in the presence of only 10 the lyase was expressed as 100%.

The pH of the reaction medium

Enzymes added	3.5	4.5	5.0
Lyase only	100	100	100
Lyase + isoamylase	136	152	150
Lyase + pullulanase	132	158	155

4. Reversible and Irreversible Inhibitors of the lyase

The reversible inhibitors, Glucose and Maltose.

25

At a substrate concentration of 10mg/ml, the activity for the lyase decreased by 19.3 % in the presence of 0.1 M glucose when amylopectin was used as substrate; the activity was not affected when glycogen was used as substrate. In the presence of 0.1 M of maltose the activity decreased by 48.8 % and 73.4%, respectively for glycogen and amylopectin.

	Substrates Concentrations	Inhibitors	
		Glucose	Maltose
5	Amylopectin 1% (2%)	19.3% (7%)	73.4% (67.2%)
	Glycogen 1% (2%)	0.000 (-)	48.8% (49.7%)

It seems that the inhibition by 0.1 M glucose is competitive as increasing the substrate from 1% to 2% decreased the inhibition from 19.3 to 7%, whereas the inhibition by 10 0.1 M maltose is non-competitive as the increase of substrate did not significantly affect the inhibition degree.

	Substrates	Glucose	Maltose
15	Amylopectin (1%)	28%	80%
	Glycogen (1%)	5%	57%

20

The reversible inhibitor deoxyjirimycin

At a final substrate concentration of 2%, the activity was decreased to 10.4% for the algal lyase in the presence of 25 μ M of deoxyjirimycin, using amylopectin as 25 substrate. At 100 μ M, the activity of the lyase was completely lost.

Irreversible Inhibitor: PCMB

Under the same assay conditions and in the presence of 2 mM PCMB, the activity 30 decreased by 60% for the lyase.

SCALE PRODUCTION OF AF

1. Production of AF using dextrin as substrate

5 The reactor contained 1000 g dextrans (obtained by treatment of starch with Termamyl to a final reducing matter of 10 %) in a final volume of 4.6 liter (HOAC-NaOAC, pH 3.9, containing 5 mM Na₂-EDTA). The reaction was initiated by adding 3 mg purified lyase. The reaction was performed at room temperature. At day 19, another batch of lyase (4 mg) was added.

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Reaction time (days)

0	1	7	13	19	24	31
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AF produced (grams)

0	18	116	195	264	500	668
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2. Using ¹⁴C-Starch for the production of ¹⁴C-AF

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The uniformly labelled ¹⁴C-starch (340 µCi obtained from Sigma) was vacuum-dried to remove the ethanol it contained and then dissolved in 2 ml water. The reaction was initiated by adding 20 µl purified lyase and 20 µl pullulanase (MegaZyme Ltd.) The reaction was performed overnight at 30 °C. At the end of the reaction, the reaction mixture was filtered using a filter with a molecular mass cut off of 10,000 to remove the enzymes and unreacted starch molecules.

30

The filtrate was applied on a Ca₂ carbohydrate column (Chrompack) using a Waters HPLC. Water was used as eluent. The flow rate was 0.5 ml/min. AF was efficiently separated from glucose and maltosaccharides. The pooled AF fractions were freeze-dried and totally 140 µCi ¹⁴C-AF was obtained.

These findings relate to an even further aspect of the present invention, namely the use of a reagent that can increase the hydrophobicity of the reaction medium (preferably an alcohol) to increase the stability and activity of the lyase according to the present invention. This increased stability leads to a increased AF yield.

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Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

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[GRINDOX as used herein is a trade mark. MAYODAN as used herein is a trade mark.]

SEQUENCE LISTING

(1) GENERAL INFORMATION

NAME OF APPLICANTS: DANISCO A/S
BUSINESS ADDRESS: Langebrogade 1
DK-1001 Copenhagen K
Denmark

TITLE OF INVENTION: ENZYME

SEQ. ID. NO. 1

SEQUENCE TYPE: NUCLEIC ACID
MOLECULE TYPE: DNA (GENOMIC)
ORIGINAL SOURCE: ALGAL
SEQUENCE LENGTH: 3279 BP
STRANDEDNESS: DOUBLE
SEQUENCE:

10	20	30	40	50	60
1 ATGTTTCC	TA CCCTGACCT	T CATAGCGCCC	A AGCGCGCTGG	C CCGCCAGCAC	C CTTTGTGGC
61 GCGGATATCC	GATCGGGCAT	TCGCATTCAA	TCCGCTCTTC	CGGCCGTGCG	CAACGCTGTG
121 CGCAGGAGCA	AACATTACAA	TGTATCCATG	ACCGCATTGT	CTGACAAGCA	AACCGCTATC
181 AGTATTGGCC	CTGACAATCC	GGACGGTATC	AACTACCAAA	ACTACGATTA	CATCCCTGTA
241 GCGGGCTTTA	CGCCCCCTCTC	CAACACCAAC	TGGTATGCTG	CCGGCTCTTC	CACTCCGGGC
301 GGCATCACCG	ACTGGACCGC	TACCATGAAT	GTCAAATTG	ACCGCATTGA	CAATCCGTG
361 TACTCCAATA	ACCATCCTGT	TCAGATTCA	GTCACGTCGT	ACAACAACAA	CAGCTTCAGG
421 ATTGCGTTCA	ACCCTGATGG	CCCCATTG	T GACGTCTCTC	GAGGACCTAT	CCTGAAACAG
481 CAACTCACTT	GGATTCGAAA	CCAGGAGCTG	GCGCAGGGAT	GTAATCCGAA	CATGAGCTTC
541 TCTCCTGAAG	GTTTTTGTC	TTTGAAACC	AAAGACCTAA	ACGTTATAAT	CTACGGCAAC
601 TGCAAGATGA	GAGTCACGAA	GAAGGGATGGC	TACCTCGTCA	TGGAGAATGA	CGAGTGCAAC
661 TCGCAATCAG	ATGGCAATAA	GTGTAGAGGA	TTGATGTACG	TTGACCGGCT	ATACGGTAAT
721 GCTATTGCTT	CCGTACAAAC	GAATTTTCAC	AAAGACACTT	CTCGAACGA	GAAATTCTAT
781 GGTGCAGGTG	AAGTCAACTG	TCGCTATGAG	GAGCAGGGTA	AGGCGCCGAC	TTATGTTCTA
841 GAACGCTCTG	GACTCGCCAT	GACCAATTAC	AATTACGACA	ACTTGAAC	CAACCAACCA
901 GACGTCGTTTC	CTCCAGGTTA	TCCCGACCAT	CCCAACTACT	ACATTCCAAT	GTACTACGCA
961 GCACCGTGGT	TGGTCGTTCA	GGGATGCGCG	GGGACATCGA	AGCAATACTC	GTACGGTTGG

1021 TTTATGGACA ATGTCTCTCA GTCGTACATG AACACTGGAG ATACGGCGTG GAACTGCGGA
1081 CAGGAAAACC TGGCATAACAT GGGCGCGCAA TACGGGCCAT TTGATCAGCA CTTTGTGTAT
1141 GGTGATGGAG ATGGCCTTGA AGATGTCGTC AAAGCGTTCT CCTTCTTCA AGGAAAGGAG
1201 TTCGAAGACA AAAAACTCAA CAAGCGTTCT GTAATGCCTC CGAAGTACGT GTTTGGTTTC
1261 TTCCAGGGTG TTTCGGTGC ACTTTCACTG TTGAAGCAGA ATCTGCCTGC CGGAGAGAAC
1321 AACATCTCAG TGCAAGAGAT TGTGGAGGGT TACCAAGGATA ACGACTACCC CTTTGAAGGG
1381 CTCGCGGTAG ATGTTGATAT GCAAGATGAT CTGCGAGTGT TTACTACCAA ACCAGAAATAT
1441 TGGTCGGCAA ACATGGTAGG CGAAGGGCGGT GATCCTAATA ACAGATCAGT CTTTGAATGG
1501 GCACATGACA GGGGCCTTGT CTGTCAGACG AACGTAACCT GCTTCTTGAG GAACGATAAC
1561 AGTGGGAAAC CATACTGAAGT GAATCAGACA TTGAGGGAGA AACAGTTGTA TACGAAGAAC
1621 GATTCTTGA ACAACACCGA TTTTGGAACT ACCTCGGATG GGCCTGGCGA TCGTACATT
1681 GGACATTTGG ACTATGGTGG TGGAGTGGAG TGTGATGCAA TCTTCCCAGA CTGGGGTCGA
1741 CCAGACGTGG CTCAATGGTG GGGAGAAAAC TACAAGAACG TGTTCAGCAT TGGTCTCGAT
1801 TTCGTGTGGC AGGATATGAC GGTACCTGCG ATGATGCCGC ACCGACTCGG TGATGCTGTC
1861 AACAAAAAATT CCGGTAGTTC GGCGCCGGGC TGGCCGAATG AGAACGATCC ATCCAACCGA
1921 CGATACAACG GGAAATCTTA TCATCCGCAA GTGCTCGTGA CCGACATGCG CTATGGTGCA
1981 GAGTATGGAA GGGAACCGAT GGTGTCTCAA CGCAACATTG ACGCCTACAC TCTTGTGAA
2041 TCTACCAGAC GGGAGGGAAAT TGTGGGAAAC GCAGACAGTT TGACCAAGTT CCGCCGCAGT
2101 TACATCATCA GTCGAGGAGG TTACATCGGT AACCAAGCATT TCGGAGGGAT GTGGGTTGGG
2161 GACAACAGTG CCACAGAACATC CTACCTCCAA ATGATGTTGG CGAACATTAT CAACATGAAT
2221 ATGTCGTGCC TCCCCCTAGT TGGCTCTGAT ATTGGCGGGT TCACCCAGTA CAATGATGCG
2281 GGCGACCCAA CCCCCGAGGA TTTGATGGTA AGATTCGTGC AGGCTGGCTG TCTGCTACCG
2341 TGGTTCAGAA ACCACTATGA CAGGTGGATT GAGTCCAAGA AGCACGGGAA GAAATACCAAG
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2461 CGCTGGCAGG AGGTTTGTA CACAGCCATG TACCAAAATG CTACCACTGG AGAGCCGATC
2521 ATCAAGGCAGG CGCCCATGTA CAACAACGAC GTCAACGTGT ATAAATCGCA GAATGATCAT
2581 TTCCCTTCTCG GTGGACATGA CGGCTATCGT ATTCTCTGCG CACCTGTTGT GCGCGAAAAT
2641 GCGACAAGTC GCGAAGTGTG CCTGCCTGTG TATAGCAAGT GGTTCAAATT CGGACCGGAC

2701 TTTGACACTA AGCCCTTGGAA AATGAGATT CAAGGAGGTC AGACGCTTTA TAATTACGCT
 2761 GCACCGCTGA ACGATTGCC GATATTGTG AGGGAAAGGGA CTATTCTTCC GACACGGTAC
 2821 ACGCTGGACG GTGTGAACAA ATCTATCAAC ACGTACACAG ACAATGATCC GCTTGATTT
 2881 GAGCTGTTCC CTCTCGAAAA CAACCAGGGC CATGGCTTGT TCTATCATGA TGATGGCGGT
 2941 GTCACCACCA ACGCTGAAGA CTTTGGCAAG TATTCTGTGA TCAGTGTGAA GGCCGCGCAG
 3001 GAAGGTTCTC AAATGAGTGT CAAGTTGAC AATGAAGTTT ATGAACACCA ATGGGGAGCA
 3061 TCGTTCTATG TTCTGTTCG TAATATGGGT GCTCCGTCTA ACATCAACGT ATCTTCTCAG
 3121 ATTGGTCAAC AGGACATGCA ACAGAGCTCC GTGAGTTCCA GGGCGCAAAT GTTCACTAGT
 3181 GCTAACGATG GCGAGTACTG GGTTGACCAG AGCACGAAC CGTTGTGGCT CAAGTTGCCT
 3241 GGTGCAGTTA TCCAAGACGC TGCGATCACT GTTCGTTGA

SEQ. ID. NO. 2

SEQUENCE TYPE: NUCLEIC ACID
 MOLECULE TYPE: DNA (GENOMIC)
 ORIGINAL SOURCE: ALGAL
 SEQUENCE LENGTH: 1712 BP
 STRANDEDNESS: DOUBLE
 SEQUENCE:

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61	CATGATTCAAG	ATCCTGACTA	CTATATTCCG	ATGTACTTTG	CGGCACCATG	GGTGATCGCA
121	CATGGATATC	GTGGCACCAAG	CGACCAGTAC	TCTTATGGAT	GGTTTTGGGA	CAATGTATCC
181	CAGTCCTACA	CAAACACTGG	CGATGATGCA	TGGGCTGGTC	AGAAGGATT	GGCGTACATG
241	GGGGCACAAAT	GTGGGCCTTT	CGATCAACAT	TTTGTGTATG	AGGCTGGAGA	TGGACTTGAA
301	GACGTTGTGA	CCGCATTCTC	TTATTTGCAA	GGCAAGGAAT	ATGAGAACCA	GGGACTGAAT
361	ATACGTTCTG	CAATGCCCTCC	GAAGTACGTT	TTCGGATTTT	TCCAAGGGGT	ATTCGGAGCC
421	ACATCGCTGC	TAAGGGACAA	CTTACCTGCC	GGCGAGAACCA	ACGTCTCTT	GGAAGAAATT
481	GTTGAAGGAT	ATCAAAATCA	GAACGTGCCA	TTTGAAGGTC	TTGCTGTGGA	TGTTGATATG
541	CAAGATGACT	TGAGAGTGTT	CACTACGAGA	CCAGCGTTT	GGACGGCAA	CAAGGTGGGG
601	GAAGGCGGTG	ATCCAAACAA	CAAGTCAGTG	TTTGAGTGGG	CACATGACAG	GGGCCTTGTC
661	TGCCAGACGA	ATGTAACTTG	CTTCTTGAAG	AACGAGAAAA	ATCCTTACGA	AGTGAATCAG

721 TCATTGAGGG AGAACGAGTT GTATACGAAG AGTGATTCCCT TGGACAACAT TGATTTGGA
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 901 AACTACAAGA AACTATTCAAG CATTGGTCTC GATTCGTCT GGCAAGATAT GACGGTACCT
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 1381 ATTGGAGGTT TCACGGAGCA TGACAAGAGA AACCCTTGCA CACCGGACTT GATGATGAGA
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 1561 TTGAGAAGTT TTGTGGAACCT CCGCTATCGC TGGCAGGAAG TGTTATACAC AGCCATGTAT
 1621 CAGAATGCTT TGAACGGGAA GCCGATCATC AAAACGGTCT CCATGTACAA CAACGATATG
 1681 AACGTCAAAG ATGCTCAGAA TGACCACTTC CT

SEQ. ID. NO. 3

SEQUENCE TYPE: ENZYME

MOLECULE TYPE: AMINO ACID

ORIGINAL SOURCE: ALGAL

SEQUENCE LENGTH: 1092 AMINO ACIDS

SEQUENCE:

	<u>5</u>	<u>10</u>	<u>15</u>
1	Met Phe Pro Thr Leu Thr Phe Ile Ala Pro Ser Ala Leu Ala Ala		
16	Ser Thr Phe Val Gly Ala Asp Ile Arg Ser Gly Ile Arg Ile Gln		
31	Ser Ala Leu Pro Ala Val Arg Asn Ala Val Arg Arg Ser Lys His		
46	Tyr Asn Val Ser Met Thr Ala Leu Ser Asp Lys Gln Thr Ala Ile		
61	Ser Ile Gly Pro Asp Asn Pro Asp Gly Ile Asn Tyr Gln Asn Tyr		
76	Asp Tyr Ile Pro Val Ala Gly Phe Thr Pro Leu Ser Asn Thr Asn		

91 Trp Tyr Ala Ala Gly Ser Ser Thr Pro Gly Gly Ile Thr Asp Trp
106 Thr Ala Thr Met Asn Val Lys Phe Asp Arg Ile Asp Asn Pro Ser
121 Tyr Ser Asn Asn His Pro Val Gln Ile Gln Val Thr Ser Tyr Asn
136 Asn Asn Ser Phe Arg Ile Arg Phe Asn Pro Asp Gly Pro Ile Arg
151 Asp Val Ser Arg Gly Pro Ile Leu Lys Gln Gln Leu Thr Trp Ile
166 Arg Asn Gln Glu Leu Ala Gln Gly Cys Asn Pro Asn Met Ser Phe
181 Ser Pro Glu Gly Phe Leu Ser Phe Glu Thr Lys Asp Leu Asn Val
196 Ile Ile Tyr Gly Asn Cys Lys Met Arg Val Thr Lys Lys Asp Gly
211 Tyr Leu Val Met Glu Asn Asp Glu Cys Asn Ser Gln Ser Asp Gly
226 Asn Lys Cys Arg Gly Leu Met Tyr Val Asp Arg Leu Tyr Gly Asn
241 Ala Ile Ala Ser Val Gln Thr Asn Phe His Lys Asp Thr Ser Arg
256 Asn Glu Lys Phe Tyr Gly Ala Gly Glu Val Asn Cys Arg Tyr Glu
271 Glu Gln Gly Lys Ala Pro Thr Tyr Val Leu Glu Arg Ser Gly Leu
286 Ala Met Thr Asn Tyr Asn Tyr Asp Asn Leu Asn Tyr Asn Gln Pro
301 Asp Val Val Pro Pro Gly Tyr Pro Asp His Pro Asn Tyr Tyr Ile
316 Pro Met Tyr Tyr Ala Ala Pro Trp Leu Val Val Gln Gly Cys Ala
331 Gly Thr Ser Lys Gln Tyr Ser Tyr Gly Trp Phe Met Asp Asn Val
346 Ser Gln Ser Tyr Met Asn Thr Gly Asp Thr Ala Trp Asn Cys Gly
361 Gln Glu Asn Leu Ala Tyr Met Gly Ala Gln Tyr Gly Pro Phe Asp
376 Gln His Phe Val Tyr Gly Asp Gly Leu Glu Asp Val Val
391 Lys Ala Phe Ser Phe Leu Gln Gly Lys Glu Phe Glu Asp Lys Lys
406 Leu Asn Lys Arg Ser Val Met Pro Pro Lys Tyr Val Phe Gly Phe
421 Phe Gln Gly Val Phe Gly Ala Leu Ser Leu Leu Lys Gln Asn Leu
436 Pro Ala Gly Glu Asn Asn Ile Ser Val Gln Glu Ile Val Glu Gly
451 Tyr Gln Asp Asn Asp Tyr Pro Phe Glu Gly Leu Ala Val Asp Val
466 Asp Met Gln Asp Asp Leu Arg Val Phe Thr Thr Lys Pro Glu Tyr
481 Trp Ser Ala Asn Met Val Gln Glu Gly Gly Asp Pro Asn Asn Arg
496 Ser Val Phe Glu Trp Ala His Asp Arg Gly Leu Val Cys Gln Thr

511 Asn Val Thr Cys Phe Leu Arg Asn Asp Asn Ser Gly Lys Pro Tyr
526 Glu Val Asn Gln Thr Leu Arg Glu Lys Gln Leu Tyr Thr Lys Asn
541 Asp Ser Leu Asn Asn Thr Asp Phe Gly Thr Thr Ser Asp Gly Pro
556 Gly Asp Ala Tyr Ile Gly His Leu Asp Tyr Gly Gly Val Glu
571 Cys Asp Ala Ile Phe Pro Asp Trp Gly Arg Pro Asp Val Ala Gln
586 Trp Trp Gly Glu Asn Tyr Lys Leu Phe Ser Ile Gly Leu Asp
601 Phe Val Trp Gln Asp Met Thr Val Pro Ala Met Met Pro His Arg
616 Leu Gly Asp Ala Val Asn Lys Asn Ser Gly Ser Ser Ala Pro Gly
631 Trp Pro Asn Glu Asn Asp Pro Ser Asn Gly Arg Tyr Asn Trp Lys
646 Ser Tyr His Pro Gln Val Leu Val Thr Asp Met Arg Tyr Gly Ala
661 Glu Tyr Gly Arg Glu Pro Met Val Ser Gln Arg Asn Ile His Ala
676 Tyr Thr Leu Cys Glu Ser Thr Arg Arg Glu Gly Ile Val Gly Asn
691 Ala Asp Ser Leu Thr Lys Phe Arg Arg Ser Tyr Ile Ile Ser Arg
706 Gly Gly Tyr Ile Gly Asn Gln His Phe Gly Gly Met Trp Val Gly
721 Asp Asn Ser Ala Thr Glu Ser Tyr Leu Gln Met Met Leu Ala Asn
736 Ile Ile Asn Met Asn Met Ser Cys Leu Pro Leu Val Gly Ser Asp
751 Ile Gly Gly Phe Thr Gln Tyr Asn Asp Ala Gly Asp Pro Thr Pro
766 Glu Asp Leu Met Val Arg Phe Val Gln Ala Gly Cys Leu Leu Pro
781 Trp Phe Arg Asn His Tyr Asp Arg Trp Ile Glu Ser Lys Lys His
796 Gly Lys Lys Tyr Gln Glu Leu Tyr Met Tyr Pro Gly Gln Lys Asp
811 Thr Leu Lys Lys Phe Val Glu Phe Arg Tyr Arg Trp Gln Glu Val
826 Leu Tyr Thr Ala Met Tyr Gln Asn Ala Thr Thr Gly Glu Pro Ile
841 Ile Lys Ala Ala Pro Met Tyr Asn Asn Asp Val Asn Val Tyr Lys
856 Ser Gln Asn Asp His Phe Leu Leu Gly Gly His Asp Gly Tyr Arg
871 Ile Leu Cys Ala Pro Val Val Arg Glu Asn Ala Thr Ser Arg Glu
886 Val Tyr Leu Pro Val Tyr Ser Lys Trp Phe Lys Phe Gly Pro Asp
901 Phe Asp Thr Lys Pro Leu Glu Asn Glu Ile Gln Gly Gly Gln Thr
916 Leu Tyr Asn Tyr Ala Ala Pro Leu Asn Asp Ser Pro Ile Phe Val

931 Arg Glu Gly Thr Ile Leu Pro Thr Arg Tyr Thr Leu Asp Gly Val
 946 Asn Lys Ser Ile Asn Thr Tyr Thr Asp Asn Asp Pro Leu Val Phe
 961 Glu Leu Phe Pro Leu Glu Asn Asn Gln Ala His Gly Leu Phe Tyr
 976 His Asp Asp Gly Gly Val Thr Thr Asn Ala Glu Asp Phe Gly Lys
 991 Tyr Ser Val Ile Ser Val Lys Ala Ala Gln Glu Gly Ser Gln Met
 1006 Ser Val Lys Phe Asp Asn Glu Val Tyr Glu His Gln Trp Gly Ala
 1021 Ser Phe Tyr Val Arg Val Arg Asn Met Gly Ala Pro Ser Asn Ile
 1036 Asn Val Ser Ser Gln Ile Gly Gln Gln Asp Met Gln Gln Ser Ser
 1051 Val Ser Ser Arg Ala Gln Met Phe Thr Ser Ala Asn Asp Gly Glu
 1066 Tyr Trp Val Asp Gln Ser Thr Asn Ser Leu Trp Leu Lys Leu Pro
 1081 Gly Ala Val Ile Gln Asp Ala Ala Ile Thr Val Arg

Number of amino acid residues: 1092

Amino acid composition (including the signal sequense):

64 Ala	14 Cys	18 His	33 Met	56 Thr
48 Arg	55 Gln	45 Ile	49 Phe	22 Trp
89 Asn	49 Glu	65 Leu	59 Pro	67 Tyr
73 Asp	94 Gly	46 Lys	73 Ser	73 Val

SEQ. ID. NO. 4

SEQUENCE TYPE: ENZYME

MOLECULE TYPE: AMINO ACID

ORIGINAL SOURCE: ALGAL

SEQUENCE LENGTH: 570 AMINO ACIDS

SEQUENCE:

5.	10	15
1 Met Thr Asn Tyr Asn Tyr Asp Asn Leu Asn Tyr Asn Gln Pro Asp		
16 Leu Ile Pro Pro Gly His Asp Ser Asp Pro Asp Tyr Tyr Ile Pro		
31 Met Tyr Phe Ala Ala Pro Trp Val Ile Ala His Gly Tyr Arg Gly		
46 Thr Ser Asp Gln Tyr Ser Tyr Gly Trp Phe Leu Asp Asn Val Ser		
61 Gln Ser Tyr Thr Asn Thr Gly Asp Asp Ala Trp Ala Gly Gln Lys		
76 Asp Leu Ala Tyr Met Gly Ala Gln Cys Gly Pro Phe Asp Gln His		
91 Phe Val Tyr Glu Ala Gly Asp Gly Leu Glu Asp Val Val Thr Ala		

106 Phe Ser Tyr Leu Gln Gly Lys Glu Tyr Glu Asn Gln Gly Leu Asn
121 Ile Arg Ser Ala Met Pro Pro Lys Tyr Val Phe Gly Phe Phe Gln
136 Gly Val Phe Gly Ala Thr Ser Leu Leu Arg Asp Asn Leu Pro Ala
151 Gly Glu Asn Asn Val Ser Leu Glu Glu Ile Val Glu Gly Tyr Gln
166 Asn Gln Asn Val Pro Phe Glu Gly Leu Ala Val Asp Val Asp Met
181 Gln Asp Asp Leu Arg Val Phe Thr Thr Arg Pro Ala Phe Trp Thr
196 Ala Asn Lys Val Gly Glu Gly Asp Pro Asn Asn Lys Ser Val
211 Phe Glu Trp Ala His Asp Arg Gly Leu Val Cys Gln Thr Asn Val
226 Thr Cys Phe Leu Lys Asn Glu Lys Asn Pro Tyr Glu Val Asn Gln
241 Ser Leu Arg Glu Lys Gln Leu Tyr Thr Lys Ser Asp Ser Leu Asp
256 Asn Ile Asp Phe Gly Thr Thr Pro Asp Gly Pro Ser Asp Ala Tyr
271 Ile Gly His Leu Asp Tyr Gly Gly Val Glu Cys Asp Ala Leu
286 Phe Pro Asp Trp Gly Arg Pro Asp Val Ala Gln Trp Trp Gly Asp
301 Asn Tyr Lys Lys Leu Phe Ser Ile Gly Leu Asp Phe Val Trp Gln
316 Asp Met Thr Val Pro Ala Met Met Pro His Arg Leu Gly Asp Pro
331 Val Gly Thr Asn Ser Gly Glu Thr Ala Pro Gly Trp Pro Asn Asp
346 Lys Asp Pro Ser Asn Gly Arg Tyr Asn Trp Lys Ser Tyr His Pro
361 Gln Val Leu Val Thr Asp Met Arg Tyr Asp Asp Tyr Gly Arg Asp
376 Pro Ile Val Thr Gln Arg Asn Leu His Ala Tyr Thr Leu Cys Glu
391 Ser Thr Arg Arg Glu Gly Ile Val Gly Asn Ala Asp Ser Leu Thr
406 Lys Phe Arg Arg Ser Tyr Ile Ile Ser Arg Gly Gly Tyr Ile Gly
421 Asn Gln His Phe Gly Gly Met Trp Val Gly Asp Asn Ser Ser Thr
436 Glu Asp Tyr Leu Ala Met Met Val Ile Asn Val Ile Asn Met Asn
451 Met Ser Gly Val Pro Leu Val Gly Ser Asp Ile Gly Gly Phe Thr
466 Glu His Asp Lys Arg Asn Pro Cys Thr Pro Asp Leu Met Met Arg
481 Phe Val Gln Ala Gly Cys Leu Leu Pro Trp Phe Arg Asn His Tyr
496 Asp Arg Trp Ile Glu Ser Lys Lys His Gly Lys Asn Tyr Gln Glu
511 Leu Tyr Met Tyr Arg Asp His Leu Asp Ala Leu Arg Ser Phe Val

526 Glu Leu Arg Tyr Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr
541 Gln Asn Ala Leu Asn Gly Lys Pro Ile Ile Lys Thr Val Ser Met
556 Tyr Asn Asn Asp Met Asn Val Lys Asp Ala Gln Asn Asp His Phe

CLAIMS

1. An enzyme comprising at least any one of the amino acid sequences shown as SEQ. ID. No.s 3 - 4, or any variant thereof.

5

2. A nucleotide sequence coding for the enzyme of claim 1.

3. A nucleotide sequence according to claim 2 wherein the nucleotide sequence is a DNA sequence.

10

4. A nucleotide sequence comprising a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of SEQ. ID. No. 1 or SEQ. ID. No. 2.

15

5. A method of preparing the sugar 1,5-D-anhydrofructose comprising treating an α -1,4-glucan with the enzyme α -1,4-glucan lyase, characterised in that enzyme is used in substantially pure form and wherein the enzyme is isolated from algae alone.

20

6. A method according to claim 5 wherein the algae is *Gracilaria* *lemaneiformis*.

7. A method according to claim 5 or claim 6 wherein the enzyme comprises at least any one of the amino acid sequences shown as SEQ. ID. No.s 3 - 4, or any variant thereof.

25

8. A method according to any preceding claim wherein the enzyme is obtained from the expression of a nucleotide sequence coding for the enzyme.

9. A method according to claim 8 wherein the nucleotide sequence is a DNA sequence.

30

10. A method according to claim 9 wherein the DNA sequence comprises a sequence that is the same as, or is complementary to, or has substantial homology with, or

contains any suitable codon substitutions for any of those of SEQ. ID. No. 1 or SEQ. ID. No. 2.

5 11. A method according to any one of claims 5 - 10 wherein if the glucan contains links other than and in addition to the α -1,4- links the α -1,4-glucan lyase is used in conjunction with a suitable reagent that can break the other links.

10 12. A method according to any one of claims 5 - 11 wherein the glucan is starch and preferably a hydrolase, preferably a glucanohydrolase, is used in conjunction with the α -1,4-glucan lyase.

15 13. The method according to claim 12 wherein the starch is used in high concentration - such as up to about 25% solution.

15 14. A method according to claim 12 or claim 13 wherein the hydrolase is at least one of pullanase or isoamylase.

20 15. A method according to any one of claims 5 to 14 wherein the α -1,4-glucan lyase is bound to a support or, more preferably, is in a dissolved form.

20 16. A method according to claim 15 wherein the enzyme is isolated and/or further purified from algae alone using a gel that is not degraded by the enzyme.

25 17. A method according to claim 16 wherein the gel is based on dextrin or derivatives thereof, preferably the gel is a cyclodextrin - more preferably β -cyclodextrin.

30 18. The method according to any one of claims 5 to 17 wherein the substrate is treated with the enzyme in the presence of a buffer.

30 19. The method according to any one of claims 5 to 17 wherein the substrate is treated with the enzyme in the presence of at least substantially pure water.

20. The method according to any one of claims 5 to 19 wherein the substrate is treated with the enzyme in the absence of a co-factor.
- 5 21. The method according to any one of claims 5 to 20 wherein the enzyme is used in combination with amylopectin or dextrin.
- 10 22. A method of preparing the sugar 1,5-D-anhydrofructose comprising treating an α -1,4-glucan with the enzyme α -1,4-glucan lyase characterised in that enzyme comprises at least any one of the amino acid sequences shown as SEQ. ID. No.s 3-4, or any variant thereof.
- 15 23. The sugar 1,5-D-anhydrofructose when prepared by the method of the present invention.
- 20 24. The use of a reagent that can increase the hydrophobicity of the reaction medium to increase the stability and activity of the GL enzyme.
- 25 25. Use of AF prepared by the method of the present invention as an anti-oxidant.
26. Use of AF prepared by the method of the present invention as a sweetener.
27. An enzyme substantially as described herein.
28. A method substantially as described herein.
- 25 29. A use substantially as described herein.

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Patents Act 1977
Examiner's report to the Comptroller under Section 17
(The Search report)

Application number
GB 9521167.8

Relevant Technical Fields		Search Examiner MR C SHERRINGTON
(i) UK Cl (Ed.N)	C3H (HC1, HB7E)	
(ii) Int Cl (Ed.6)	C12N 9/88, 15/60	Date of completion of Search 2 JANUARY 1996
Databases (see below)		Documents considered relevant following a search in respect of Claims :-
(i) UK Patent Office collections of GB, EP, WO and US patent specifications.		1 TO 23, 25, 26
(ii) ONLINE: WPI, CLAIMS, DIALOG/BIOTECH		

Categories of documents

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Category	Identity of document and relevant passages	Relevant to claim(s)
X	WO 95/10618 A2 (DANISCO A/S) whole document	1 to 4, 8 to 10, 16, 17
X	WO 95/10616 A2 (DANISCO A/S) whole document	1 to 23, 25, 26
X	WO 94/09122 A1 (ALGATECH AB) whole document	1 to 4, 8 to 10, 16
X	Biochim Biophys Acta 1993, 1156, 313-320 alpha-1 ₄ -Glucan lyase, a new class of starch/glycogen degrading enzyme; I Efficient...	5, 6, 23
A	Planta 1993, 191(1), 137-142 alpha-1, 4-Glucan lyase, a new class of starch/glycogen degrading enzyme, II. Subcellular...	1
A	Phytochemistry 1988, 27, 3401-3403 Fungal enzymic activity degrading 1, 4-alpha-D-glucans to 1, 5-D-anhydrofructose	5, 6, 23

Databases: The UK Patent Office database comprises classified collections of GB, EP, WO and US patent specifications as outlined periodically in the Official Journal (Patents). The on-line databases considered for search are also listed periodically in the Official Journal (Patents).

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